Veterinary Immunology and Immunopathology 126 (2008) 362–366

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Short communication

Cellular and cytokine responses in feathers of chickens vaccinated against Marek's disease

M.F. Abdul-Careem [a], D.B. Hunter [a], S. Shanmuganathan [a], H.R. Haghighi [a], L. Read [a], M. Heidari [b], S. Sharif [a,*]

[a] Department of Pathobiology, University of Guelph, Guelph, Ontario, Canada
[b] United States Department of Agriculture, Agriculture Research Service, Avian Disease and Oncology Laboratory, East Lansing, MI 48823, United States

ARTICLE INFO

Article history:
Received 2 June 2008
Received in revised form 25 June 2008
Accepted 3 July 2008

Keywords:
Chicken
Marek's disease
Vaccine
CD8+ T cell
Interferon

ABSTRACT

In Marek's disease virus infection, feather follicle epithelium (FFE) constitutes the site of formation of infectious virus particles and virus shedding. The objective of this study was to characterize cellular and cytokine responses as indicators of cell-mediated immune response in FFE and associated feather pulp following immunization against Marek's disease. Analysis of feather tips collected between 4 and 28 days post-immunization (d.p.i.) from chickens vaccinated post-hatch with either CVI988/Rispens or herpesvirus of turkeys revealed that replication of these vaccine viruses started at 7 d.p.i., peaked by 21 d.p.i., and subsequently, showed a declining trend. This pattern of viral replication, which led to viral genome accumulation in feather tips, was associated with infiltration of T cell subsets particularly CD8+ T cells into the feather pulp area and the expression of cytokine genes such as interferon-γ, which is an indication of elicitation of cell-mediated immune responses at the site of virus shedding.

1. Introduction

Marek's disease (MD) manifests mainly as lymphoproliferative and immune suppressive conditions in susceptible chickens (Calnek, 2001). The causative agent, Marek's disease virus, MDV (currently known as *Gallid herpesvirus-2*) belongs to the genus *Mardivirus*. The other two species of the genus are *Gallid herpesvirus-3* and *Meleagrid herpesvirus-1* (herpesvirus of turkeys or HVT) are naturally occurring nononcogenic species. Strains belonging to these three species are used as monovalent, bivalent or trivalent vaccines (Churchill et al., 1969; Okazaki et al., 1970; Rispens et al., 1972; Witter and Schat, 2003).

MDV infection in the skin and associated feather follicle epithelium (FFE) leads to the production of enveloped infectious virus, which is shed along with feathers and dander (Calnek et al., 1970; Beasley et al., 1970; Heidari et al., 2007). For this reason, the FFE is important in transmission of MDV and, hence, epidemiology of MD. However, it has been demonstrated that MD vaccines are unable to significantly reduce virus load in FFE (Eidson et al., 1971; Abdul-Careem et al., 2007) and in the environment (Islam and Walkden-Brown, 2007).

Recently, we found that infection with a very virulent (vv) strain of MDV results in initiation of host responses in feather tips characterized by the expression of cytokine genes, predominantly IFN-γ and infiltration of T cell subsets, namely CD4+ and CD8+ T cells into feather pulp, which was associated with a significant increase in viral replication (Abdul-Careem et al., 2008a). It is not known whether the commonly used MD vaccines elicit host responses similar to vvMDV in feather tips. The objective of

* Corresponding author at: Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1, Canada. Tel.: +1 519 824 4120x54641; fax: +1 519 824 5930.
E-mail address: shayan@uoguelph.ca (S. Sharif).

0165-2427/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetimm.2008.07.003

the study was, therefore, to investigate host responses in feather tips following vaccination against MD.

2. Materials and methods

2.1. Vaccines

Two MD vaccines were used to immunize chickens. CVI988 strain is an attenuated MDV currently categorized under the species *Gallid herpesvirus-2* and HVT is a naturally occurring turkey herpesvirus categorized as *Meleagrid herpesvirus-1* species. These vaccines were supplied by Merial Canada Inc. (Baie D'Urfe, Quebec, Canada).

2.2. Experimental design

Thirty, 1-day-old specific pathogen-free chicks were randomly divided into three equal groups; two vaccinated groups (CVI988 or HVT vaccination), and the control group. The chickens were vaccinated subcutaneously on the day of hatch as has been recommended by the vaccine manufacturer (Baie D'Urfe, Quebec, Canada). The control group was mock vaccinated with the vaccine diluent. Feather tips were collected at 4, 7, 10, 14, 21 and 28 days post-immunization (d.p.i.). On each of the 6 sampling days, 5 chickens per group were sampled.

2.3. DNA and RNA extraction and reverse transcription

DNA and RNA extraction from feather tips was carried out using Trizol reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) as has been described previously (Abdul-Careem et al., 2006b). Reverse transcription of RNA also has been described (Abdul-Careem et al., 2006b). The template for reverse transcription consisted of 2 μg of RNA (1 μg was used in the case of RNA extracted from the feather tips of control and HVT immunized chickens sampled at 4 d.p.i.).

2.4. Primers

Previously published primers were used for the absolute or relative quantification of expression of target genes (MDV glycoprotein (gB), HVT gB, IFN-α, IFN-γ) and β-actin that acted as the reference gene (Abdul-Careem et al., 2006b, 2008a,b; Brisbin et al., 2007).

2.5. Real-time RT-PCR and PCR techniques

All the DNA and cDNA preparations were tested in real-time PCR and RT-PCR assays, respectively, in LightCycler® thermocycler, version 3.5 or LightCycler® 480 thermocycler (Roche Diagnostics GmbH, Mannheim, State of Baden-Wurttemberg, Germany) as has been described previously (Abdul-Careem et al., 2006b, 2008b).

2.6. Immunohistochemistry technique

Feather tips of three animals per group per time point were frozen and the immunohistochemistry technique was employed for visualizing T cell subsets (CD4+ and CD8+) according to the previously described methods (Abdul-Careem et al., 2008a).

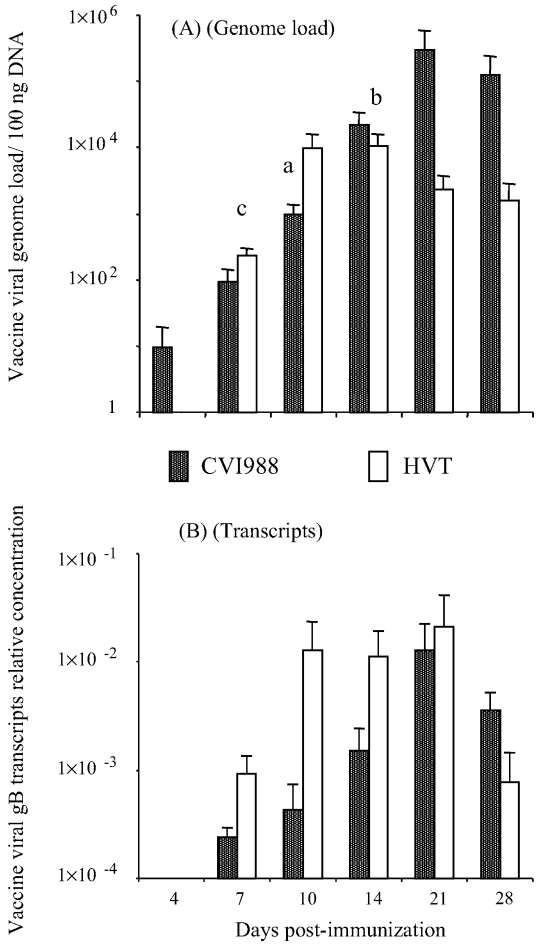

Fig. 1. Genome load (A) and gB transcripts (B) of CVI988 and HVT vaccines in feather tips. The groups were as follows; CVI988 = chickens received the CVI988 vaccine on the day of hatch, HVT = chickens received the HVT vaccine on the day of hatch. The difference in genome load and viral transcripts between observations was analyzed by *t*-test and comparisons were considered significant at $P \leq 0.05$. a = significant when compared to CVI988 genome load quantified on 4 and 7 d.p.i., b = significant when compared to HVT genome load quantified on 7 d.p.i. and c = significant when compared to HVT genome load quantified on 4 d.p.i.

2.7. Data analysis

Cells counted in five fields of 40× magnification for each chicken were averaged and subjected to statistical analysis. Quantification of genome load of CVI988 and HVT vaccines and expression of cytokine genes by real-time PCR and RT-PCR was done as has been described previously (Abdul-Careem et al., 2006a,b). Statistical analysis of data was done using the *t*-test. Comparisons were considered significant at $P \leq 0.05$.

3. Results and discussion

DNA extracted from feather tips originated from the vaccinated chickens was analyzed by real-time PCR for

CVI988 and HVT genome load (Fig. 1A). In the present study, the pattern of CVI988 genome accumulation in feather tips closely agrees with the observations made by Baigent et al. (2005, 2006) in that CVI988 genome was quantifiable from 7 d.p.i. with a peak at 21 d.p.i., followed by a decline in genome load. Similar to the viral genome load, CVI988 viral transcripts (Fig. 1B) gradually increased beginning from 7 d.p.i., peaked by 21 d.p.i. and then declined thereafter ($P > 0.05$). Though there is a lack of information on the HVT genome load in feather tips, Islam and Walkden-Brown (2007) showed that HVT genome could be detected and quantified in dust collected from poultry house environment starting at 7 d.p.i.; then HVT genome load was increased by 28 d.p.i. and became steady thereafter. In agreement with this observation, our study showed that the HVT genome in feather tips was quantifiable at 7 d.p.i., but the peak of HVT genome in feather tips was much earlier than that quantified in poultry house environment.

An increased infiltration of CD8+ T cells was observed in response to vaccination at 7 d.p.i. and onwards (Fig. 2). The infiltration of CD8+ T cells was observed mainly in the feather pulp area and not in the surrounding FFE. Our previous observations have shown that in response to vvMDV, both CD4+ and CD8+ T cells infiltrate into the tissue (Abdul-Careem et al., 2008a). However, in the present study, as illustrated in Fig. 3A and B, only the infiltration of CD8+ T cells was significant in the vaccinated chickens compared to the controls ($P \leq 0.05$). This suggests a potential role for CD8+ T cells in clearing virus-infected cells in FFE. T cells, particularly CD8+ T cells (Omar et al., 1998) may play a role in vaccine-induced immune response against MD. In agreement with this CD8+ T cell-mediated immune function, the number of circulating CD8+ T cells increases following vaccination with HVT (Quere et al., 2005). Similar to HVT, CVI988 could also induce the expansion of both T cell subsets in the spleen (Gimeno et al., 2004). Furthermore, the studies conducted by Morimura et al. (1998, 1999) indicated a clear function of CD8+ T cells in response to CVI988 vaccination.

Relative concentrations of IFN-γ and IFN-α genes in the feather tips of vaccinated and unvaccinated chickens are

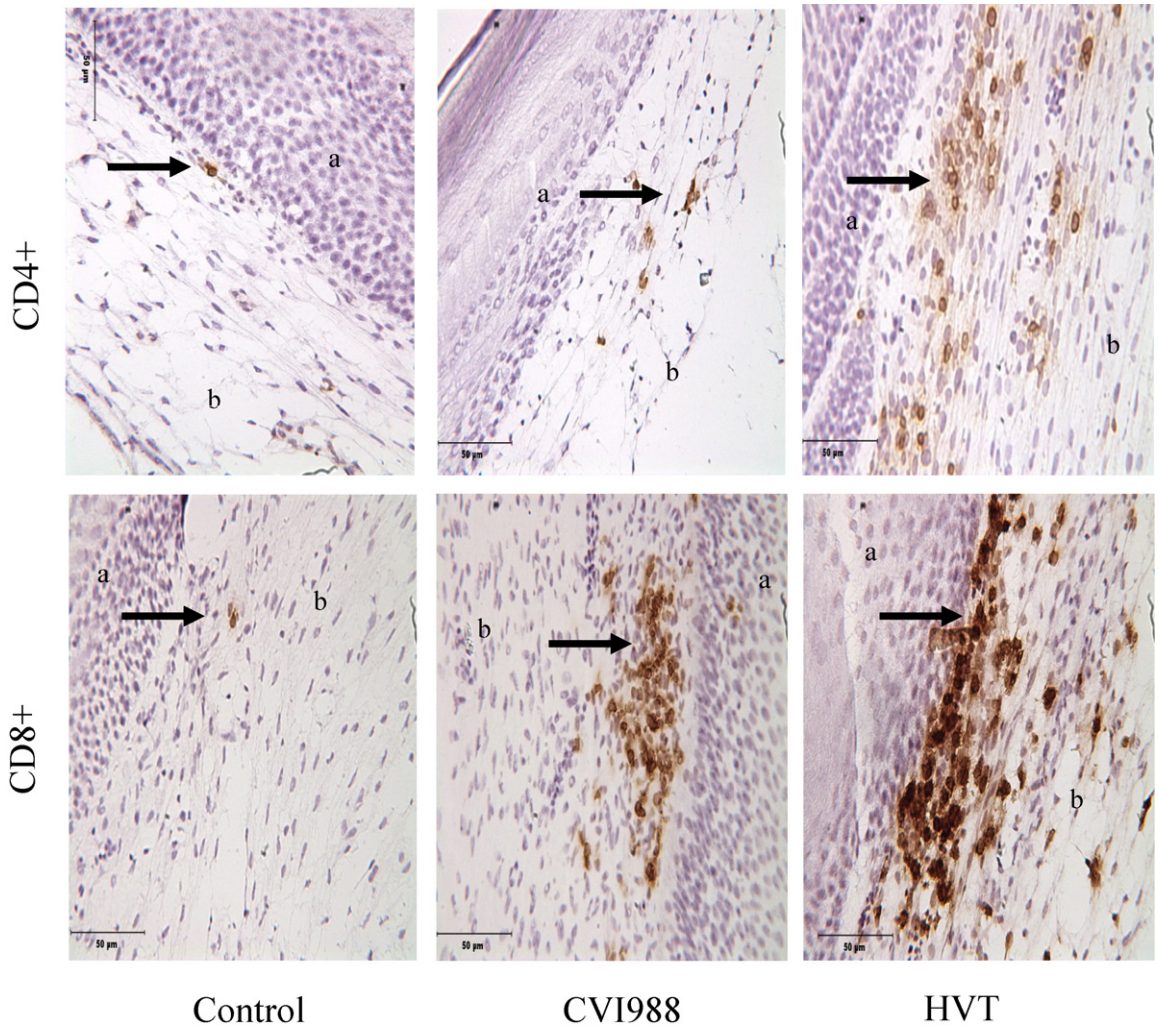

Fig. 2. Infiltration of T cell subsets in longitudinal sections of feather proximal ends in vaccinated and unvaccinated chickens. The groups were same as those described for Fig. 1 a=feather follicle epithelium, b = feather pulp.

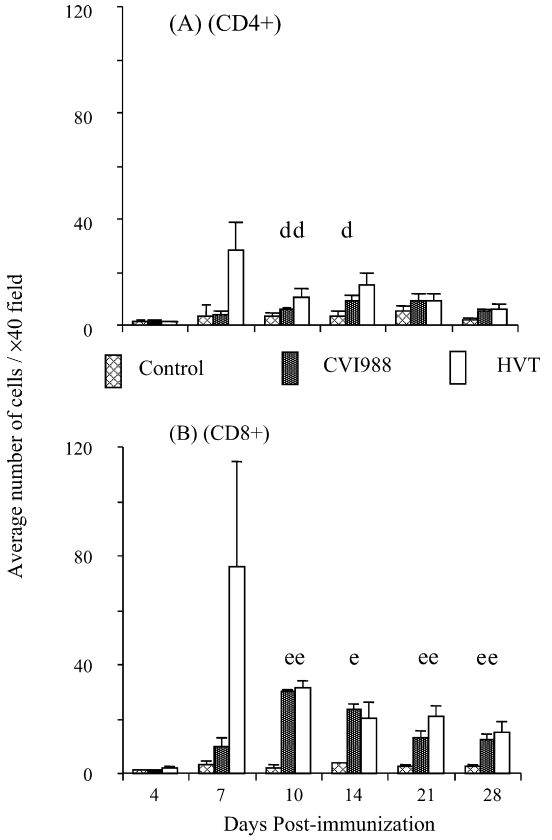

Fig. 3. Distribution of T cell subsets in feather proximal ends of vaccinated and unvaccinated chickens. Group mean number of CD4+ (A) and CD8+ (B) T cells per 40× microscopic fields are presented and the error bars represent standard error of the mean. d = significantly low when compared to the CD8+ T cells in the same group, e = significantly high when compared to the CD8+ T cell counts in the unvaccinated controls.

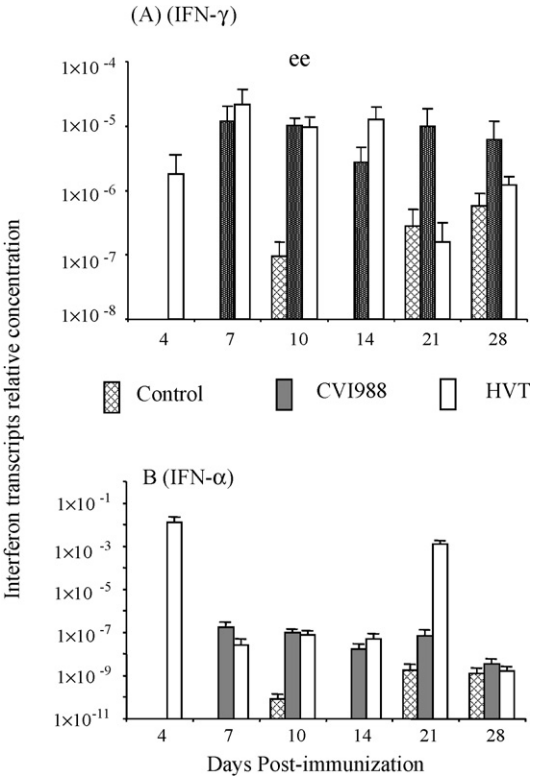

Fig. 4. Expression of IFNs in feather proximal ends of chickens vaccinated with CVI988 and HVT vaccines and unvaccinated controls. (A) and (B) show IFN-γ and IFN-α mRNA expression, respectively. The groups were same as those described for Fig. 1. Error bars represent standard error of the mean. The difference in IFN transcripts between groups was assessed by *t*-test and comparisons were considered significant at $P \leq 0.05$. e = significantly high when compared to the gene expression in the unvaccinated controls.

illustrated in Fig. 4A and B, respectively. In response to CVI988 and HVT vaccination, the expression of IFN-γ gene showed a significant increase at 10 d.p.i. ($P \leq 0.05$). Compared to the strong expression of IFN-γ gene in feather tips of chickens infected with vvMDV (Abdul-Careem et al., 2008b), it appeared that there was a relatively small, albeit statistically significant, increase in the expression of IFN-γ gene in feather tips of vaccinated birds. Others have also shown the expression of IFN-γ gene in tissues other than feather tips. The expression of IFN-γ gene in splenocytes in response to HVT infection has been shown (Djeraba et al., 2002). Also, IFNs have been shown to curtail replication of CVI988 and HVT *in vitro* (Levy et al., 1999). The present study provides evidence that both CVI988 and HVT elicit the expression of IFN-γ gene in feather tips similar to that described previously for vvMDV (Abdul-Careem et al., 2008a). The up-regulation of IFN-γ gene in feather tips in response to vaccination with CVI988 and HVT could be related to the antiviral activity of this cytokine mediated through macrophage activation and nitric oxide (NO) synthesis, similar to what has been shown for MDV infection (Lee, 1979; Xing and Schat, 2000). In addition to these effects, IFN-γ could play a role in cytotoxicity against virus-infected cells mediated by CD8+ cytotoxic T cells (Whitmire et al., 2005).

Since feathers are involved in shedding of infectious virulent MDV irrespective of vaccination and, consequently, transmission of the virus to susceptible chickens leading to lymphoproliferation (Calnek et al., 1970; Beasley et al., 1970), the importance of the present study is twofold. Both CVI988 and HVT vaccine viral strains are capable of inducing an immune response characterized by the expression of IFN-γ and infiltration of T cell subsets particularly CD8+ T cells into feather pulp, which is similar to the host response shown for vvMDV infection (Abdul-Careem et al., 2008a). However, the host responses stimulated in feather tips by CVI988 and HVT vaccines differ from those observed previously with vvMDV infection (Abdul-Careem et al., 2008a). In our previous study using a vvMDV strain, we noted a significant infiltration of both T cell subsets as well as a consistent up-regulation of expression of IFN-γ gene in feather tips between 7 and 14 d.p.i. However, vaccination with HVT or CVI988 only led to infiltration of CD8+ T cells into feather pulp and a transient up-regulation of IFN-γ expression at 10 d.p.i.

In conclusion, we have shown that MD vaccine viruses, particularly CVI988 and HVT strains replicate in FFE and elicit host responses characterized by the expression of IFN-γ and CD8+ T cell infiltration. These findings provide a basis for future studies aimed at the development of vaccines that could reduce shedding of virulent MDV from infected chickens.

Acknowledgements

M.F. Abdul-Careem is a recipient of a Canada Graduate Scholarship from NSERC and a postgraduate scholarship supplement from CPRC. This work was funded by PIC and NSERC.

References

Abdul-Careem, M.F., Hunter, D.B., Lambourne, M.D., Read, L.R., Parvizi, P., Sharif, S., 2008b. Expression of cytokine genes following pre- and post-hatch immunization with herpesvirus of turkeys. Vaccine 26, 2369–2377.

Abdul-Careem, M.F., Hunter, B.D., Nagy, E., Read, L.R., Sanei, B., Spencer, J.L., 2006a. Development of a real-time PCR assay using SYBR green chemistry for monitoring Marek's disease virus genome load in feather tips. J. Virol. Methods 133, 34–40.

Abdul-Careem, M.F., Hunter, B.D., Parvizi, P., Haghighi, H.R., Thanthrige-Don, N., Sharif, S., 2007. Cytokine gene expression patterns associated with immunization against Marek's disease in chickens. Vaccine 25, 424–432.

Abdul-Careem, M.F., Hunter, B.D., Sarson, A.J., Mayameei, A., Zhou, H., Sharif, S., 2006b. Marek's disease virus-induced transient paralysis is associated with cytokine gene expression in the nervous system. Viral Immunol. 19, 167–176.

Abdul-Careem, M.F., Hunter, B.D., Sarson, A.J., Parvizi, P., Haghighi, H.R., Read, L., Heidari, M., Sharif, S., 2008a. Host responses are induced in feathers of chickens infected with Marek's disease virus. Virology 370, 323–332.

Baigent, S.J., Smith, L.P., Currie, R.J., Nair, V.K., 2005. Replication kinetics of Marek's disease vaccine virus in feathers and lymphoid tissues using PCR and virus isolation. J. Gen. Virol. 86, 2989–2998.

Baigent, S.J., Smith, L.P., Nair, V.K., Currie, R.J., 2006. Vaccinal control of Marek's disease: current challenges, and future strategies to maximize protection. Vet. Immunol. Immunopathol. 112, 78–86.

Beasley, J.N., Patterson, L.T., McWade, D.H., 1970. Transmission of Marek's disease by poultry house dust and chicken dander. Am. J. Vet. Res. 31, 339–344.

Brisbin, J.T., Zhou, H., Gong, J., Sabour, P., Akbari, M.R., Haghighi, H.R., Yu, H., Clarke, A., Sarson, A.J., Sharif, S., 2007. Gene expression profiling of chicken lymphoid cells after treatment with *Lactobacillus acidophilus* cellular components. Dev. Comp. Immunol. 32, 563–574.

Calnek, B.W., 2001. Pathogenesis of Marek's disease virus infection. Curr. Top. Microbiol. Iimmunol. 255, 25–55.

Calnek, B.W., Adldinger, H.K., Kahn, D.E., 1970. Feather follicle epithelium: a source of enveloped and infectious cell-free herpesvirus from Marek's disease. Avian Dis. 14, 219–233.

Churchill, A.E., Payne, L.N., Chubb, R.C., 1969. Immunization against Marek's disease using a live attenuated virus. Nature 221, 744–747.

Djeraba, A., Musset, E., Bernardet, N., Le Vern, Y., Quere, P., 2002. Similar pattern of iNOS expression, NO production and cytokine response in genetic and vaccination-acquired resistance to Marek's disease. Vet. Immunol. Immunopathol. 85, 63–75.

Eidson, C.S., Fletcher, O.J., Kleven, S.H., Anderson, D.P., 1971. Detection of Marek's disease antigen in feather follicle epithelium of chickens vaccinated against Marek's disease. J. Natl. Cancer Inst. 47, 113–120.

Gimeno, I.M., Witter, R.L., Hunt, H.D., Reddy, S.M., Reed, W.M., 2004. Biocharacteristics shared by highly protective vaccines against Marek's disease. Avian Pathol. 33, 59–68.

Heidari, M., Fitzgerald, S.D., Zhang, H.M., Silva, R.F., Lee, L.F., Dunn, J.R., 2007. Marek's disease virus-induced skin leukosis in scaleless chickens: tumor development in the absence of feather follicles. Avian Dis. 51, 713–718.

Islam, A., Walkden-Brown, S.W., 2007. Quantitative profiling of the shedding rate of the three Marek's disease virus (MDV) serotypes reveals that challenge with virulent MDV markedly increases shedding of vaccinal viruses. J. Gen. Virol. 88, 2121–2128.

Lee, L.F., 1979. Macrophage restriction of Marek's disease virus replication and lymphoma cell proliferation. J. Immunol. 123, 1088–1091.

Levy, A.M., Heller, E.D., Leitner, G., Davidson, I., 1999. Effect of native chicken interferon on MDV replication. Acta Virol. 43, 121–127.

Morimura, T., Cho, K., Kudo, Y., Hiramoto, Y., Ohashi, K., Hattori, M., Sugimoto, C., Onuma, M., 1999. Anti-viral and anti-tumor effects induced by an attenuated Marek's disease virus in CD4- or CD8-deficient chickens. Arch. Virol. 144, 1809–1818.

Morimura, T., Ohashi, K., Sugimoto, C., Onuma, M., 1998. Pathogenesis of Marek's disease (MD) and possible mechanisms of immunity induced by MD vaccine. J. Vet. Med. Sci. 60, 1–8.

Okazaki, W., Purchase, H.G., Burmester, B.R., 1970. Protection against Marek's disease by vaccination with a herpesvirus of turkeys. Avian Dis. 14, 413–429.

Omar, A.R., Schat, K.A., Lee, L.F., Hunt, H.D., 1998. Cytotoxic T lymphocyte response in chickens immunized with a recombinant fowlpox virus expressing Marek's disease herpesvirus glycoprotein B. Vet. Immunol. Immunopathol. 62, 73–82.

Quere, P., Rivas, C., Ester, K., Novak, R., Ragland, W.L., 2005. Abundance of IFN-alpha and IFN-gamma mRNA in blood of resistant and susceptible chickens infected with Marek's disease virus (MDV) or vaccinated with turkey herpesvirus; and MDV inhibition of subsequent induction of IFN gene transcription. Arch. Virol. 150, 507–519.

Rispens, B.H., van Vloten, H., Mastenbroek, N., Maas, H.J., Schat, K.A., 1972. Control of Marek's disease in the Netherlands. I. Isolation of an avirulent Marek's disease virus (strain CVI 988) and its use in laboratory vaccination trials. Avian Dis. 16, 108–125.

Whitmire, J.K., Tan, J.T., Whitton, J.L., 2005. Interferon-gamma acts directly on CD8+ T cells to increase their abundance during virus infection. J. Exp. Med. 201, 1053–1059.

Witter, R.L., Schat, K.A., 2003. Diseases of poultry. In: Saif, Y.M. (Ed.), Marek's Disease. Iowa State University Press, Ames, IA, USA, pp. 407–467.

Xing, Z., Schat, K.A., 2000. Inhibitory effects of nitric oxide and gamma interferon on in vitro and in vivo replication of Marek's disease virus. J. Virol. 74, 3605–3612.